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Histamine and cholesterol lowering abilities of lactic acid bacteria isolated from artisanal Pico cheese

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ABSTRACT

Aims

This study was designed to select lactic acid bacteria with histamine and cholesterol reducing abilities to be use as potential probiotics.

Methods and Results

Thirty strains of lactic acid bacteria isolated from an artisanal raw milk cheese were screened for their abilities to degrade histamine, reduce cholesterol and hydrolyze bile salts. Strains were also screened for safety and probiotic traits, such as resistance to gastrointestinal conditions, adhesion to Caco-2 cells. resistance to antibiotics and presence of virulence genes. Two *Lactobacillus paracasei* strains presented high cholesterol- and histamine-lowering abilities, tested negative for the presence of virulence genes and showed susceptibility to most important antibiotics. These strains were also shown to possess desirable *in vitro* probiotic properties, revealed by tolerance to gastrointestinal conditions and high adhesion to intestinal cells.

Conclusions

Among the screened strains, *Lb. paracasei* L3C21M6 revealed the best cholesterol and histamine reducing abilities together with desirable probiotic and safety features to be used in food applications.

Significance and Impact of the Study

The strain L3C21M6 is a good candidate for use as a probiotic with histamine-degrading activity and cholesterol lowering effect. In addition, this strain could be use in dairy foods to prevent histamine food poisoning.

Keywords

Lactic acid bacteria; probiotic; cholesterol; histamine; cheese

Introduction

The probiotic market is predicted to reach $57.4 billion by 2022 driven largely by consumers' interest in preventative healthcare and the desire for natural products (Allied Market Research, 2016). Probiotics can be consumed in different forms being encapsulated in pills or incorporated into dairy foods including yogurt, cheese and other fermented foods (Granato et al. 2010). Most probiotics belong to the lactic acid bacteria (LAB) group, and the most commonly used are members of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* species (Parvez et al. 2006).

According to the FAO/WHO (2002), probiotic food products are generally fermented foods containing a sufficient amount of viable and active "living nonpathogenic microorganisms" (bacteria or yeasts) that, when ingested, are able to confer numerous health benefits on the host. Key health-promoting properties of probiotic bacteria rely on their ability to survive passage through the gastrointestinal tract (GIT), which depends on several factors including their capacity to tolerate the acidic pH of the stomach, as well as bile and digestive enzymes (Gobbetti et al. 2010) and to adhere to intestinal epithelial cells and/or mucus (Marco et al. 2006).

Different studies in humans have provided evidence of the beneficial effects of probiotics. Consumption of probiotics has been shown to stimulate the growth of beneficial microorganisms and reduce pathogen load, to alleviate certain intolerances (such as lactose intolerance), and to prevent or reduce allergies (Isolauri 2001; Tang et al. 2015). Clinical evidence also exists for probiotic efficacy in several conditions including irritable bowel syndrome, constipation, diarrhea, bacterial vaginosis, hepatic steatosis and treatment of high cholesterol (Plaza-Diaz et al. 2014; Puebla-Barragan and Reid, 2019). In addition, certain LAB may improve the health-promoting potential of probiotic foods by degrading anti-nutritional compounds such as biogenic amines and cholesterol (Chiang and Pan 2012; Trautvetter et al. 2012).

The consumption of fermented foods, and especially cheese, with high concentrations of biogenic amines, can cause food poisoning such as histamine intoxication, mimicking an allergic reaction (EFSA 2011). In individuals with histamine intolerance, ingestion of food with normal contents of histamine causes histamine-mediated symptoms, and has been associated with a number of inflammatory and neoplastic diseases, such as Crohn disease, ulcerative colitis, allergic enteropathy, food allergy and colorectal neoplasms (Maintz and Novak 2007; Ladero et al. 2010). Therefore, the use of LAB capable of decreasing the concentration of histamine in foods and in the GIT should lead to a reduction in incidence of histamine poisoning and the relief of adverse reactions in individuals with histamine intolerance.

High blood cholesterol (hypercholesterolemia) is a risk factor for cardiovascular disease, which remains one of the largest causes of death worldwide (Ishimwe et al. 2015). Probiotic supplements have been shown to significantly reduce serum total cholesterol in humans (Wang et al., 2018). The mechanisms proposed for these cholesterol-lowering effects include deconjugation of bile salts by bacterial bile salt hydrolase (Yıldız et al. 2011). Therefore, the ability of LAB to hydrolyse bile salts has been included among the criteria for probiotic strain selection (Peres et al. 2014).

In a previous study, LAB strains were isolated from artisanal Pico cheese and evaluated on basis of technological, safety and consumer preference (Domingos-Lopes et al. 2017). Thirty strains with QPS status (*Lactobacillus*, *Lactococcus* and *Leuconostoc* genus) showed absence of beta-hemolysis, DNAse, gelatinase and histamine production, in addition to suitable technological features. The objective of the present study is to identify potential probiotic strains from this group. Therefore, we evaluated the *in vitro* probiotic potential of these LAB strains concerning their safety and ability to degrade histamine, reduce cholesterol and hydrolyze bile salts. In addition,

Materials and methods

Bacteria strains and growth conditions

A total of 30 LAB strains were previously isolated from Pico cheese (Domingos-Lopes et al. 2017) and identified as *Leuconostoc mesenteroides* (4)*, Leu. citreum* (1)*, Lactococcus lactis* (2), *Lc. garvieae* (1)*, Lactobacillus plantarum (*5), *Lb. paraplantarum* (1)*, Lb. paracasei* subsp. *paracasei* (15) and *Lb. otakiensis* (1). Stock cultures were kept at -80 °C in 50% (v/v) glycerol and propagated twice in MRS broth (Biokar, Beauvais, France) with 1% (v/v) of inoculum, aerobically at 30 °C, for 24 h, before use.

Assessment of the ability of the LAB strains to degrade histamine

The ability of LAB strains to degrade histamine was tested in a model system similar to that previously described by Dapkevicius et al. (2000). LAB strains were tested for their ability to degrade histamine in diamine oxidase (DO) broth, consisting of 1 g l^{-1} glucose, 3 g l^{-1} yeast extract, 3 g l^{-1} tryptone, 5 g l^{-1} NaCl, 1 g l^{-1} Tween 80, 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.038 g l^{-1} $MnSO_4 \cdot H_2O$, 0.08 g l^{-1} $FeSO_4.7H_2O$ and 0.05 g l^{-1} histamine dihydrochloride, pH 6.0. The strains were previously grown in MRS broth at 30 °C for 24 h, harvested by centrifugation (5,000 × *g*, 10 min, 4°C), washed with phosphate buffer saline (PBS, pH 7.2), and incubated in DO broth (1%) at 37 ºC, for 30 h.

The histamine concentration was determined by the spectrofluorometric method of Shore (1971). Samples of 1 ml of inactivated broth were added to 9 ml of perchloric acid (0.4 mol l^{-1}) and centrifuged at 2,000 × *g*. The supernatant (2 ml) was added to a mix of 1-butanol, 0.25 ml NaOH and 0.75g NaCl (5 ml). The whole mixture was shaken for 5 min and centrifuged for 1 min at 2,000 × *g*. Two ml of the upper layer was added to n-heptane (4 ml) and 3 ml HCl (0.1 mol l^{-1}).

After 15 min shaking, the whole mixture was centrifuged again and 2 ml of the under layer (aqueous-acid phase) was taken for the *o*-phthaldialdehyde (OPT)-reaction. The OPT reaction was done with the addition of 0.4 ml of NaOH (1mol l^{-1}) to the aqueous acid phase (2 ml) and 0.1 ml of OPT-reagent (1 g OPT in 100 ml methanol). This mixture was mixed and allowed to stand at room temperature for 4 min. The reaction was stopped with 0.2 ml HCl (3 mol l^{-1}) and histamine content was measured with a fluorescence spectrophotometer (Fluostar Omega, BMG), at a 360 nm excitation and 450 nm emission wavelengths. All determinations were carried out in triplicate. The percentage of histamine degradation was calculated as follows: % Histamine degradation = [1 - (residual histamine in the DO broth)/(histamine content of the control DO broth)]×100. Results are expressed as the average of three independent experiments.

Determination of cholesterol-lowering activity

The LAB strains were assessed for cholesterol-lowering activity in MRS liquid broth supplemented with cholesterol, according to Damodharan et al. (2015). MRS-CHO broth was prepared with MRS broth, 0.05% cysteine-HCl and 500 μg ml^{-1} (w/v) of cholesterol (Sigma Chemical Co., USA). The MRS-CHO broth was inoculated with bacterial culture (2%) and incubated at 37 ºC for 24 h. The cholesterol concentration in spent medium was estimated by enzymatic colorimetric method (CHOD-PAP cholesterol kit, NS Biotec, Egypt), following the manufacturer's protocol. Cholesterol content of inoculated MRS-CHO broth was compared to uninoculated MRS-CHO broth (control) and the percentage of cholesterol removal was calculated as follows: % cholesterol reduction = [1 - (residual cholesterol in the broth)/(initial cholesterol content of the medium)]×100. Results are expressed as the average of three independent experiments.

Bile salts deconjugation assay

Bile salts deconjugation was measured based on the procedure of (Solieri et al. 2014). Cultures were screened for bile salt hydrolase (BSH) activity by spotting 10 ml of a culture suspension onto BSH screening medium which consisted of MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA; Sigma Aldrich, Milan Italy) and 0.37 g l^{-1} of $CaCl_2$. Plates were incubated at 37 ºC for 48 h. A positive result was interpreted as the presence of precipitated bile salts around colonies (opaque halo). The diameters of the precipitation zones

were measured to evaluate BSH activity. Results are expressed as the average of three independent experiments.

Screening for probiotic properties

Resistance to gastric acidity

The methods used and described below were adapted from Argyri et al. (2013). Bacterial cells from overnight cultures were harvested (10 000 × *g*, 5 min, 4 °C), washed twice with sterile PBS (pH 7.3), before being re-suspended in 1 ml of PBS, and diluted (1:100) in PBS solution adjusted to pH 2.5 to reflect stomach acidity. Resistance was assessed in terms of viable colony counts and enumerated in duplicate on MRS agar (Biokar Diagnostics) after incubation at 37 °C for 0, 0.5, 1, 2, and 3 h, reflecting the time spent by food in the stomach. Results are expressed as the average of four independent experiments.

Resistance to bile acid and pancreatin

The effect of bile acid and pancreatin on strains was determined as reported by Silva et al. (2015). Bacterial cells from overnight cultures were harvested (10,000 × *g*, 5 min, 4 °C), washed twice with PBS buffer (pH 7.3), before being re-suspended in PBS solution (pH 7.3), containing 0.3% (w/v) of bile salts and 0.1% (w/v) of pancreatin. Resistance was assessed in terms of viable colony counts and enumerated in duplicate on MRS agar (Biokar Diagnostics) after incubation at 37 °C for 0, 0.5, 1, 2, and 3 h, reflecting the time spent by food in the small intestine. Results are expressed as the average of four independent experiments.

Adherence to Caco-2 cell line

The strains were examined for their ability to adhere to human colon carcinoma (Caco-2) cells. Bacterial adherence to differentiated Caco-2 cells (15 days) was tested as described by Argyri et al. (2013) with minor modifications. Caco-2 cells (European Collection of Cell Cultures, ECACC 09042001) were grown in culture plates pre-coated with rat-tail collagen (Collagen, Type I Solution, C3867 Sigma), in Dulbecco's Modified Eagle's Medium (D6429, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, F2442 Sigma), 1% nonessential amino acids (M7145, Sigma) and 1% gentamicin (G1397, Sigma). The cell-line was incubated at 37 °C in a humid atmosphere of 5% CO_2 and transferred into 24 well tissue culture plates at a seeding density of 10^4 cells. The medium in the wells was replaced with fresh medium every 2-3 days for 15 days, until monolayers

formed. Prior to experiments, all bacterial cultures were grown overnight in MRS at 37 °C, washed twice with PBS and added to each well (approximately 10^6 CFU ml^{-1}). Following co-incubation for 2 h at 37 °C, cells were washed three times with sterile PBS to remove any non-adherent bacteria. Then, 0.1 ml of trypsin-EDTA solution was added, and the mixture was incubated for 10 min at 37 °C in 5% CO_2. Each monolayer was disrupted by repeated pipetting with 0.4 ml of 0.25% Triton X-100. The number of associated bacteria was determined by plating appropriate dilutions of the lysate onto agar plates and incubating for 48 hours at 37 °C. All the experiments were repeated three times independently and, in each experiment, the strains were tested in duplicate.

Safety evaluation

Susceptibility to antibiotics

Susceptibility to antibiotics was performed by the disc diffusion method, according to CLSI (2016). Antibiotic discs (Oxoid, England) were used to determine the susceptibility of the strains to 22 antibiotics: β-lactams (combinations) - amoxicillin/clavulanic acid (20/10 μg/disc); penicillins - carbenicillin (100 μg/disc), penicillin (10 IU/disc) and piperacillin (100 μg/disc); cephalosporins - ceftazidime (30 μg/disc), ceftriaxone (30 μg/disc), cefotaxime (30 μg/disc) and cephalotin (30 μg/disc); aminoglycosides - gentamicin (10 μg/disc), kanamycin (30 μg/disc), netilmicin (30 μg/disc), streptomycin (10 μg/disc) and tobramycin (10 μg/disc); amphenicols - chloramphenicol (30 μg/disc), lincosamides - clindamycin (2 μg/disc); macrolides - erythromycin (15 μg/disc), quinolones - nalidixic acid (30 μg/disc) and ofloxacin (5 μg/disc); rifamycins - rifampicin (5 μg/disc), sulfonamides - trimethoprim/sulfamethoxazole (1.25/23.75 μg/disc), tetracyclines - tetracycline (30 μg/disc); and glycopeptides - vancomycin (30 μg/disc). Analyses were done in duplicate. The discs were placed onto the surface of inoculated Mueller-Hinton (AES, France) agar plates seeded with LAB strains that had been previously grown in MRS broth for 24 h - 48 h at 30°C. After incubation at 30°C for 24 h, the diameter of inhibition halos around the disks was measured with a digital calliper (Absolute Digimatic Caliper, Mitutoyo, USA), to assess the susceptibility or resistance of the examined isolates. Each isolate was characterized as sensitive (S) or resistant (R) according to the inhibition zone diameters in agreement with the Clinical and Laboratory Standards Institute tables (CLSI 2016).

Presence of virulence genes

The strains were tested for the presence of virulence genes: gelatinase (*gelE*), hyaluronidase (*hyl*), aggregation substance (*asa1*), enterococcal surface protein (*esp*), cytolisin (*cylA*), endocarditis antigen, (*efaA*), collagen adhesion (*ace*), antibiotic resistance genes (*vanA* and *vanB,* both related to vancomycin resistance), and genes for histidine decarboxylase (*hd2*), as reported previously by Ribeiro et al. (2014). *E. faecalis* (Ribeiro et al. 2014) and *E. faecium* (Lopez et al. 2009) strains were used as positive controls in the corresponding PCR reactions.

Statistical analysis

Data are expressed as means ± SEM. Principal Component Analysis (PCA) with varimax rotation was done to group strains based on functional properties (degradation of histamine, cholesterol reduction after 24h and 48h, and bile salts deconjugation). *In vitro* adhesion to Caco-2 cells by different strains were compared and analyzed by One-Way ANOVA. Differences were considered statistically significant at $P < 0.05$. Statistical analyses were performed with SPSS software package (IBM SPSS Statistics 20, IBM Corporation, New York, USA).

Results

Functional properties

Thirty autochthonous strains were previously screened for their ability to produce histamine by decarboxylation of histidine, and all tested negative for histamine production (Domingos-Lopes et al. 2017). The histamine degrading, cholesterol reducing and bile salts deconjugating abilities of these strains are summarized in Table 1. Sixteen strains present high histamine reduction potential as they degraded histamine to over 50% of its initial concentration in culture medium. Among them, three strains of *Lb. paracasei* (L2A1K8, L2B1K8 and L3B21R2), one *Leu. mesenteroides* (L3A21M4) and one *Lb. plantarum* (strain 11, L2C21E8) were most active in removing histamine (>58% reduction).

The ability of the strains to reduce cholesterol *in vitro* also varied between strains and among species (Table 1). Cholesterol removal increased from a range of 15–58% after 24 h, and a range of 20–66% after 48 h. The highest percentage of cholesterol reduction was achieved by *Lb. paracasei* strain L3B1M2 (22), lowering cholesterol by 54% and 66%, after 24 h and 48 h, respectively. *Lb. paracasei* L3B21R1 strain (23) also presented a high degree of cholesterol removal after 24 h and 48 h (58% and 60%, respectively) in association with moderate histamine

lowering ability (46%). Likewise, *Leu. mesenteroides* strain L2B21E3 (2) presented a high degree of cholesterol removal after 48 h (60%) and moderate histamine degradation capacity (41%). Moreover, strains *Lb. paracasei* L2B1K8 (20), L3B21R2 (24) and L3C21M6 (28) presented high capacity to reduce cholesterol (>50%, after 48h) in association to high capacity to degrade histamine (>50%).

Deconjugation of bile salts was also observed for all the strains tested. All the 30 LAB strains screened showed the precipitation zone ranging from 12 to 15 mm (Table 1). Curiously, the species/strains showing high bile salt hydrolase (BSH) activities (14-15 mm) presented lower ability for cholesterol removal (strains 3 to 9, 17 and 30).

Principal component analysis was applied to group the strains according to their different capacities to degrade cholesterol, histamine and bile salts. The first two axes accounted for 74% of the total variation and distributed the strains into five distinct groups (A, B, C, D and E, Figure 1a). Principal component 1 (PC-1) accounted for 49% of variation (Fig. 1b) and was mainly responsible for grouping the strains according to cholesterol reduction after 24 h and 48 h. Principal component 2 (PC-2) accounted for 25% of variation and was responsible for grouping the strains according to histamine reduction and BSH activity (Fig. 1b). Cluster A is the largest group (14 strains) and includes strains with high histamine reduction ability, but reduced capacity to degrade cholesterol (Fig. 1a). Strains clustered in group B (strains 20, 24 and 28) presented high capacity to reduce both cholesterol and histamine, but smaller BSH activity (Fig. 1a). Strains clustered in the group C (strains 22 and 23) showed high capacity for cholesterol reduction but lower capacity for histamine reduction and bile salt deconjugation (Fig. 1a). Cluster D contains five strains (1, 13, 14, 21 and 26) showing all together low values of histamine degradation, cholesterol removal and BSH activity (Fig. 1a). Finally, group E includes the strains with reduced capacity to degrade both histamine and cholesterol, but high BSH activity (strains 2, 4, 5, 6, 9 and 10, Fig. 1a).

Probiotic properties

The survival of LAB strains under low pH (2.5) is presented in Fig.2a. Generally, all strains showed poor resistance to the gastric environment as demonstrated by the rapid loss of viability after exposure to pH 2.5 (Fig. 2a). *Leu. mesenteroides* (L2B21E3), *Lb. plantarum* L2A21R1 and *Lb. paracasei* strains (L2A1K8, L3B21R1, L3B21R2 and L3C21M6) showed

highest survival to acidic conditions for 2 h. Only one strain (strain 9, *Lb. plantarum* LA21R1) was detectable (1.6 log CFU ml^{-1}) after 3 h of exposure to the acidic conditions.

In contrast to poor resistance to acidic conditions, most strains were found to be tolerant to bile salts and pancreatin (Fig. 2b). In fact, even after 3 h of exposure, most strains retained their viability. In contrast, two strains (*Leu. mesenteroides* L3A21M4 and *Lb. paracasei* L2B1K8) demonstrated an important loss in viability after 3 h and one *Lb. paracasei* (L3B21K4) was highly sensitive to bile salts and pancreatin (undetectable after two hours of exposition). Other strains, including *Leu. mesenteroides* L2B21E3 (strain 2) and *Lb. paracasei* L3B1M2 and L3B21R1 (22 and 23), exhibited survival greater than 80% after 3 h in the presence of bile salts and pancreatin (Fig. 2b).

The probiotic potential of strains was also evaluated for their ability to colonize the GIT epithelial cells. The efficiency of each strain's capacity to adhere to a Caco-2 cell line is presented in Fig. 2c. In general, all strains displayed high adhesion capacity to Caco-2 cells and no significant differences ($P > 0.05$) were found between strains.

Safety

The antibiotic susceptibility of the LAB strains is presented in Fig. 3. All strains were sensitive to amoxicillin/clavulanic acid, chloramphenicol, carbenicillin, penicillin, piperacillin and tetracycline. Most of the strains were resistant to aminoglycosides (kanamycin, streptomycin and tobramycin), with the exception of netilmicin. Few strains were resistant to erythromycin (*Lc. garvieae* L3B1M8), cefalotin (*Lb. paraplantarum* L2B21R5 and *Lb. paracasei* L3B21R2), ofloxacin (*Lb. paracasei* L2B21R1a) and rifampicin (*Leu. mesenteroides* L2B21E3 and *Lc. lactis* L3A21M1). Vancomycin resistance was found in three strains of *Leu. mesenteroides* (strains L2A21E7, L3A21M4 and L3C21R7), two *Lb. plantarum* (strains L2B21R1b and L3C1E8) and four *Lb. paracasei* (strains L2A1K8, L2B1K8, L3A21R8 and L3B1K1).

The results of evaluation of the virulence potential of the strains are presented in Table 2. None of the strains harbored the gene for the hyaluronidase (*hyl*). The gene for endocarditis (*efaA*) was more common, detected in the majority of strains, with the exception of one *Leu. mesenteroides* (strain 4, L3C21R7) and seven *Lb. paracasei* (strains 16, 17, 18, 19, 22, 26 and 28, L2A1K8, L2A21K5, L2B21R1a, L2B21R3, L3B1M2, L3B1K1 and L3C21M6, respectively). Most of the strains harbour at least one virulence gene, but six strains (strain 4, *Leu. mesenteroides*

L3C21R7 and strains 16, 18, 19, 22, 28, *Lb. paracasei* L2A1K8, L2B21R1a, L2B21R3, L3B1M2, L3C21M6, respectively) tested negative for all the virulence genes studied.

Discussion

Large amounts of histamine and other biogenic amines can be found in a variety of foods, particularly in fish, cheese and other fermented food products (Doeun et al. 2017; Park et al. 2019). Several studies have described the use of bacteria for reducing biogenic amine concentrations in foods, especially in fermented products (Dapkevicius et al. 2000; Zaman et al. 2010; Lee et al. 2016). Dietary-derived biogenic amines are mainly metabolized in the digestive tract by diamine oxidase (DAO) (Kawashima et al. 2011). Reduced DAO activity in individuals can lead to histamine intolerance. Therefore, the presence of probiotic bacteria with biogenic amine-degrading activities may be useful to help detoxification, particularly when intestinal DAO is reduced.

Among biogenic amines, histamine is the most likely to cause food poisoning (Izquierdo-Casas et al. 2019). In this study, high percentages of histamine degradation (over 50%) were detected for sixteen strains, making these good candidates for probiotic use (Table 1). Remarkably, all strains showed the ability to degrade histamine, with a large variation between the strains. Similarly, Zaman et al (2009) found that all the bacteria isolated from fish sauce which did not produce biogenic amines, had the ability to degrade histamine, although with different efficiencies (in the range from 5% to 60%). The percentages of histamine degradation were slighter higher in the present study (29% to 60%), but these results were comparable to the study of Dapkevicius et al. (2000). In their study, lactobacilli isolated from naturally fermented fish pastes were found to reduce histamine by 20–56% in culture media enriched with histamine, but the percentage of positive strains was very low. However, the different source of LAB may be responsible by this result, since scombroid fish is frequently a source of histamine-food poisoning. Studies concerning histamine degradation by LAB isolated from cheese are scarce. The few studies concerning histamine degradation are related to LAB isolated from fermented fish because this food, in particular scombroid fish, is one of the main sources of histamine food-poisoning. Although from a different matrix, LAB isolated from cheese share similarities with fish isolates, included the ability to degrade histamine. In a different study, Leuschner et al. (1998) found 27 histamine-degrading bacteria out of 64 LAB isolated from food, but did not specified the food

source. Herrero-Fresno et al. (2012) also found a reduced number of *Lb. casei* strains (17 out of 157) presenting over 25% of histamine degradation rate, but they were isolated from high biogenic amine cheeses.

In the present study, 14 strains were clustered in group A of the PCA plot (Fig. 1a) that showed high histamine reduction ability, although reduced capacity to degrade cholesterol. This cluster was dominated by *Lb. paracasei* (9 strains), although other species were also included, such as *Lb. plantarum, Lc. garvieae, Lc. lactis* and *Leu. mesenteroides*. These strains have the potential to be used as probiotics in individuals with reduced DAO activity, but also in food fermentations to reduce histamine content. The mechanism involved in histamine reduction by these strains is unknown, but other studies described the amino-oxidase activity of food-fermenting microorganisms as a way to reduce biogenic amines (Leuschner et al. 1998).

Cholesterol-lowering ability is also an important trait for probiotic bacteria. Some studies have revealed a relationship between consumption of fermented dairy products and a reduction of serum cholesterol levels in humans and animals (Andrade and Borges 2009; Hjerpsted et al. 2011; Yadav et al. 2019). The hypocholesterolemic effects of fermented foods were attributed to the presence of bacteria able to remove cholesterol in the small intestine (Huang et al. 2014; Michael et al. 2017; Zhang et al. 2017). The cholesterol-lowering efficacy of probiotic bacteria was shown to be highly strain-specific (Papanikolaou et al. 2012; Zhang et al. 2013; Saravanan et al. 2015; Nami et al. 2018). We observed similar results in this study, as LAB strains removed the cholesterol *in vitro* at variable levels (20–66%, after 48 h). However, only two strains of *Lb. paracasei* (L3B1M2 and L3B21R1) were clustered in the group displayed high cholesterol-lowering efficacy (group C, Fig.1a). These strains showed high levels of cholesterol reduction (>50%) after 24 h (Table 1).

Cholesterol removal by bacteria has been shown to improve with the addition of bile salts to culture media (Taranto et al. 1997). Previous reports on cholesterol reduction by *Lactobacillus* strains showed high levels (28–86%) of cholesterol reduction in liquid medium supplied with 0.3% bile oxgall (Yıldız et al. 2011; Papanikolaou et al. 2012). In the present work, some strains showed high levels of cholesterol removal (>50%), although no bile salts were used in the culture media.

The mechanisms of cholesterol-lowering activity of LAB include its adsorption to the cell wall and assimilation into the cell membrane. The incorporation of cholesterol into the cell membrane has been proposed as a tool to improve survival of lactobacilli in the gastrointestinal

tract (Dambekodi and Gilliland 1998; Horáčková et al. 2018). Other mechanisms of cholesterol reduction by bacteria involve the conversion of cholesterol to coprostanol and co-precipitation of cholesterol with deconjugated bile salts (Yıldız et al. 2011; Gérard 2014). In the present study, cholesterol reduction was not observed when cells were not removed by centrifugation (data not shown). Therefore, cholesterol adsorption to the cell wall or membrane is likely to be the mechanism involved in cholesterol removal from fermentation media by these strains.

Hydrolysis of bile salts by the gut microbiota has been proposed by several authors as an additional mechanism for reducing cholesterol (Gérard 2014; Michael et al. 2017). The removal of cholesterol by gut bacteria was revealed to be linked to the BSH activity of the cells, releasing unconjugated bile acids that induce cholesterol co-precipitation (Taranto et al. 1997; Liong and Shah 2005). In this study, all 30 isolates screened showed BSH activity as confirmed by the precipitation zone, in agreement with other studies (Saravanan et al. 2015). However, no correlation was found between cholesterol reduction and BSH activity by the cells. Similarly, Dambekodi and Gilliland (1998) showed no relationship between the ability to deconjugate bile salts and cholesterol assimilation by bacteria. Bile salts deconjugation might play a vital role in sustaining the stability of the gut microbiota. According to Smet et al. (1995), BSH activity allows lactobacilli to persist under harsh intestinal bile stress. In addition, Ma et al. (2019) showed that strains exhibiting greater BSH ability *in vitro* have also higher potential to decrease serum cholesterol levels *in vivo*. However, high BSH deconjugation activity may produce detrimental effects for the host. Indeed, if this hydrolysis is more extensive in the gut, it could lead to colon cancer, gallstones, and other GIT diseases (McGarr et al. 2005).

PCA plot was effective in showing the differences among strains concerning phenotypic features (Fig. 1). *Lb. paracasei* strains clustered in group B (strains 20, 24 and 28) of the PCA plot displayed the highest functional properties, because they showed higher activities for cholesterol and histamine removal, while they presented satisfactory BSH activity (12.5-13.5 mm). Among the strains clustered in this group, strain 20 (*Lb. paracasei* L2B1K8) was the least tolerant to acid and bile salts and pancreatin. In addition, this strain also presented the lowest adhesion to Caco-2 cells ($<10^4$ UFC cm^{-2}). The remaining two strains (24 and 28) showed similar tolerance to GIT conditions and adhesion to Caco-2 cells did not differ significantly ($P > 0.05$). However, strain 28 (*Lb. paracasei* L3C21M6) held the highest probiotic traits, including both cholesterol, histamine and BSH activities. Both these strains were considered safe, as they were sensitive to most of the antibiotics tested, with the exception of cephalosporins and aminoglycosides. However,

aminoglycoside resistance has been described as an intrinsic feature of *Lactobacillus* species (Campedelli et al. 2019). In addition, *Lb. paracasei* L3C21M6 tested negative for all virulence genes. Although expression of genes was not assessed, the presence of virulence genes is a safety issue due to the potential transfer to pathogenic bacteria in the gut.

In a previous study, strain *Lb. paracasei* L3C21M6 was showed to hold important enzymatic properties for dairy application, including high esterase/lipase activities, extracellular proteolytic activity and strong production of diacetyl from citrate (Domingos-Lopes et al. 2017). This strain was also displayed the ability to produced a pleasant aroma/flavour in experimental fresh cheeses (Domingos-Lopes et al. 2017).

In conclusion, the present study revealed that *Lb. paracasei* L3B21R2 and L3C21M6 strains isolated from Pico artisanal cheese possessed good cholesterol and histamine-lowering abilities and bile salt hydrolase activities. These strains were not only tolerant to gastrointestinal conditions but also presented high adhesion to intestinal cells and were considered safe. The probiotic potential of *Lb. paracasei* L3C21M6 is complemented with a previous report of desirable technological and sensory characteristics in experimental cheeses. Therefore, *Lb. paracasei* L3C21M6 could be considered as a potential probiotic LAB strain for food and technological applications.

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Conflict of Interest

No conflict of interest declared.

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Table 1. Histamine degradation (%), cholesterol removal (%) after 24 and 48 h, and bile salt hydrolase activity (diameters of the precipitation zones) of bacterial strains used in the study.

N	Species	Strain	Accession No.	Histamine degradation (%)	Cholesterol removal (%)		Bile salt hydrolase activity (mm)
					24 h	48 h	
1	*Leu. mesenteroides*	L2A21E7	KM079353	29.2 ± 5.8	38 ± 6.5	45 ± 2.9	12.0
2	*Leu. mesenteroides*	L2B21E3	KM079354	41.0 ± 7.1	32 ± 5.6	60 ± 3.2	13.5
3	*Leu. mesenteroides*	L3A21M4	KM079355	59.6 ± 8.1	32 ± 6.2	37 ± 4.1	15.0
4	*Leu. mesenteroides*	L3C21R7	KM079356	43.4 ± 8.9	19 ± 2.6	38 ± 7.1	15.0
5	*Leu. citreum*	L3C1E7	KM079357	35.5 ± 9.3	46 ± 8.1	47 ± 5.5	14.5
6	*Lc. lactis lactis*	L3B1M7	KM079358	44.4 ± 8.3	28 ± 8.5	38 ± 5.9	15.0
7	*Lc. garvieae*	L3B1M8	KM079359	55.6 ± 9.1	27 ± 4.9	43 ± 5.0	14.5
8	*Lc. lactis*	L3A21M1	KF193424	50.3 ± 3.3	36 ± 6.5	45 ± 4.5	14.5
9	*Lb. plantarum*	L2A21R1	KM103931	36.6 ± 5.0	31 ± 8.8	39 ± 5.9	14.5
10	*Lb. plantarum*	L2B21R1b	KM103932	39.0 ± 5.0	27 ± 8.9	39 ± 1.3	13.5
11	*Lb. plantarum*	L2C21E8	KM103933	59.2 ± 8.5	30 ± 3.2	48 ± 8.9	14.0
12	*Lb. plantarum*	L3A21R6	KM103934	52.7 ± 9.4	34 ± 9.7	39 ± 8.4	13.5
13	*Lb. plantarum*	L3C1E8	KM079361	43.8 ± 6.9	37 ± 6.2	42 ± 9.8	13.0
14	*Lb. paraplantarum*	L2B21R5	KM079360	40.8 ± 8.5	32 ± 5.4	34 ± 9.5	13.0
15	*Lb. paracasei*	L2A21R9	KM096813	54.2 ± 4.2	33 ± 5.8	34 ± 6.5	13.5
16	*Lb. paracasei*	L2A1K8	KM096814	58.4 ± 9.2	37 ± 2.6	39 ± 1.3	12.0
17	*Lb. paracasei*	L2A21K5	KM096815	55.8 ± 4.5	31 ± 2.4	39 ± 8.4	14.5
18	*Lb. paracasei*	L2B21R1a	KM096816	57.8 ± 2.8	35 ± 3.8	37 ± 0.8	13.0
19	*Lb. paracasei*	L2B21R3	KM096817	52.3 ± 8.3	24 ± 6.9	34 ± 5.9	12.5
20	*Lb. paracasei*	L2B1K8	KM096818	58.1 ± 6.2	39 ± 8.1	52 ± 5.5	12.5
21	*Lb. paracasei*	L3A21R8	KM096819	40.3 ± 6.2	38 ± 5.1	47 ± 2.5	13.0
22	*Lb. paracasei*	L3B1M2	KM096820	40.8 ± 9.5	54 ± 7.0	66 ± 2.5	13.0
23	*Lb. paracasei*	L3B21R1	KM096821	46.0 ± 9.4	58 ± 6.0	60 ± 7.1	12.5
24	*Lb. paracasei*	L3B21R2	KM096822	59.2 ± 5.5	42 ± 7.3	51 ± 5.5	12.5

25	*Lb. paracasei*	L3B21R7	KM096823	52.0 ± 9.7	15 ± 8.0	20 ± 9.0	12.5
26	*Lb. paracasei*	L3B1K1	KM096824	45.1± 8.0	21 ± 2.1	49 ± 5.5	12.5
27	*Lb. paracasei*	L3B21K4	KM096825	50.9 ± 7.7	21 ± 0.8	36 ± 9.7	13.0
28	*Lb. paracasei*	L3C21M6	KM096826	55.0 ± 5.8	47 ± 3.8	56 ± 0.4	13.5
29	*Lb. paracasei*	L3C1K8	KM096827	46.4 ± 8.2	24 ± 4.7	32 ± 0.8	13.5
30	*Lb. otakiensis*	L3C1R1	KM096828	55.3 ± 8.8	23 ± 4.8	31 ± 1.7	14.5

Table 2. Incidence of virulence genes in LAB strains. The virulence genes tested were gelatinase (*gelE*), hyaluronidase (*hyl*), aggregation substance (*asa1*), enterococcal surface protein (*esp*), cytolisin (*cylA*), endocarditis antigen (*efaA*), collagen adhesion (*ace*), resistance to vancomycin (*vanA* and *vanB*) and histidine decarboxylase (*hdc2*).

LAB Strains	Virulence genes*							Antibiotic resistance genes*		Histamine genes*
	gelE	*hyl*	*asa1*	*esp*	*cylA*	*efaA*	*ace*	*vanA*	*vanB*	*hdc2*
Leu. mesenteroides										
L2A21E7	-	-	-	-	+	+	-	-	-	-
L2B21E3	-	-	+	-	-	+	+	-	-	+
L3A21M4	-	-	-	-	-	+	+	-	-	-
L3C21R7	-	-	-	-	-	-	-	-	-	-
Leu. citreum										
L3C1E7	-	-	-	-	-	+	-	-	-	-
Lc.lactis										
L3B1M7	-	-	-	-	-	+	-	-	-	-
L3A21M1	-	-	-	-	-	+	-	-	-	-
Lc. garvieae										
L3B1M8	-	-	-	-	-	+	-	-	-	-
Lb. plantarum										
L2A21R1	-	-	-	-	-	+	+	-	-	-
L2B21R1b	-	-	-	-	-	+	-	-	-	-
L2C21E8	-	-	-	-	-	+	-	-	-	-
L3A21R6	-	-	-	-	-	+	-	-	-	-
L3C1E8	-	-	-	-	-	+	+	-	-	-
Lb. paraplantarum										
L2B21R5	-	-	-	-	-	+	-	-	-	-
Lb. paracasei										
L2A21R9	+	-	+	+	+	+	+	-	-	-
L2A1K8	-	-	-	-	-	-	-	-	-	-
L2A21K5	-	-	-	-	-	-	-	+	-	-
L2B21R1a	-	-	-	-	-	-	-	-	-	-
L2B21R3	-	-	-	-	-	-	-	-	-	-
L2B1K8	-	-	-	-	-	+	-	-	-	-

L3A21R8	-	-	-	-	-	+	-	-	+	-
L3B1M2	-	-	-	-	-	-	-	-	-	-
L3B21R1	-	-	-	-	-	+	-	-	-	-
L3B21R2	-	-	-	-	+	+	-	-	-	-
L3B21R7	-	-	-	-	+	+	+	-	-	-
L3B1K1	-	-	+	-	-	-	-	-	-	-
L3B21K4	-	-	-	-	-	+	-	-	-	-
L3C21M6	-	-	-	-	-	-	-	-	-	-
L3C1K8	+	-	+	+	-	+	+	-	+	-
Lb. otakiensis										
L3C1R1	-	-	+	-	-	+	+	-	-	-

* Positive (+) and negative (-) results for virulence genes.

Fig. 1

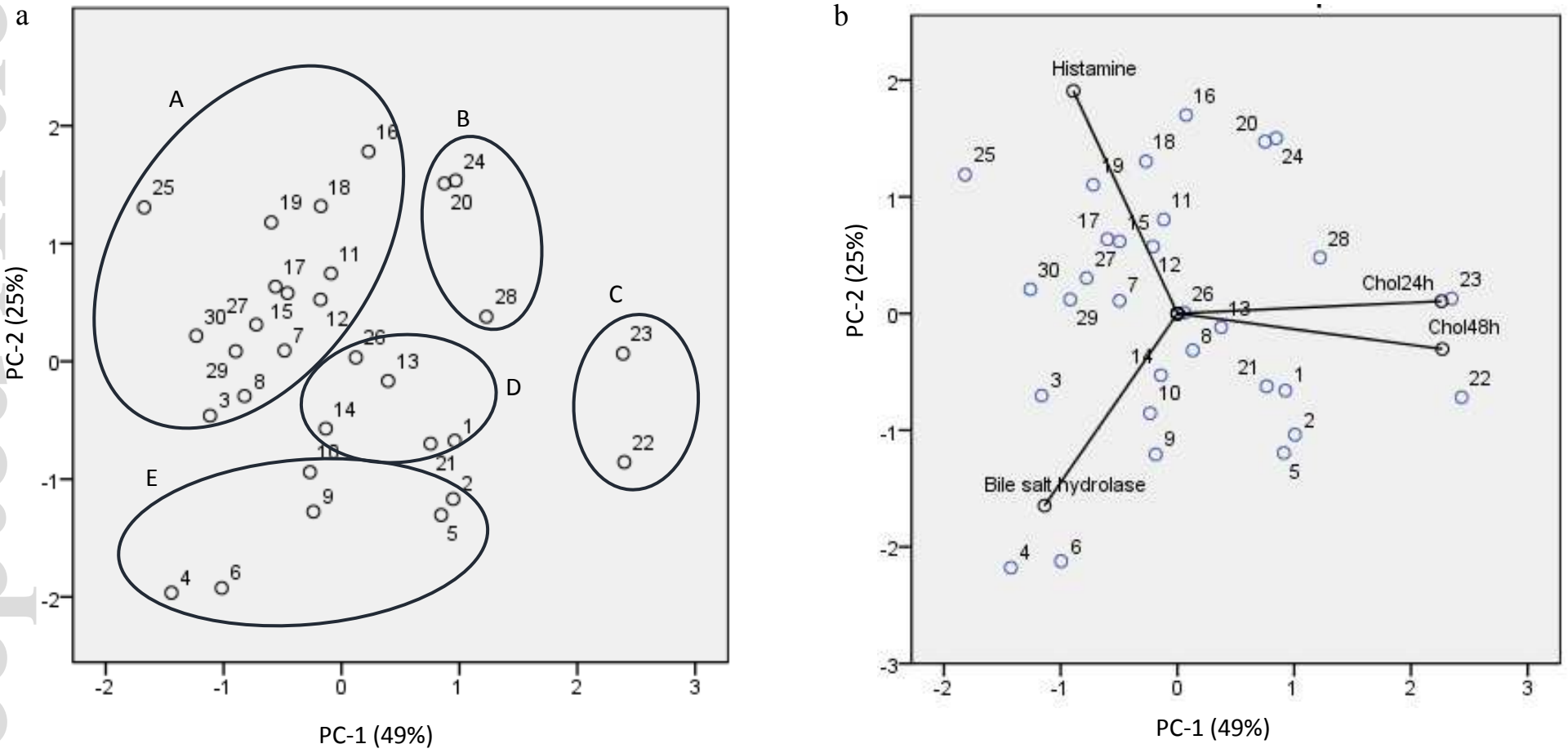
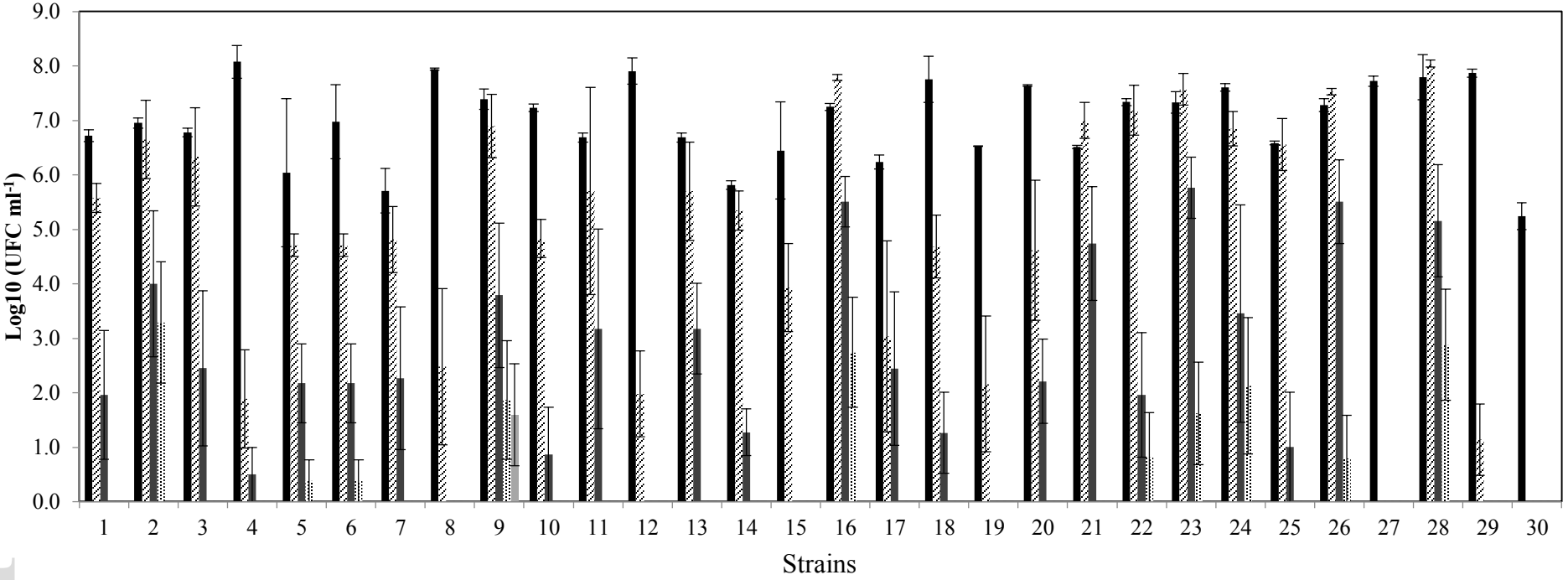


Fig. 2

A



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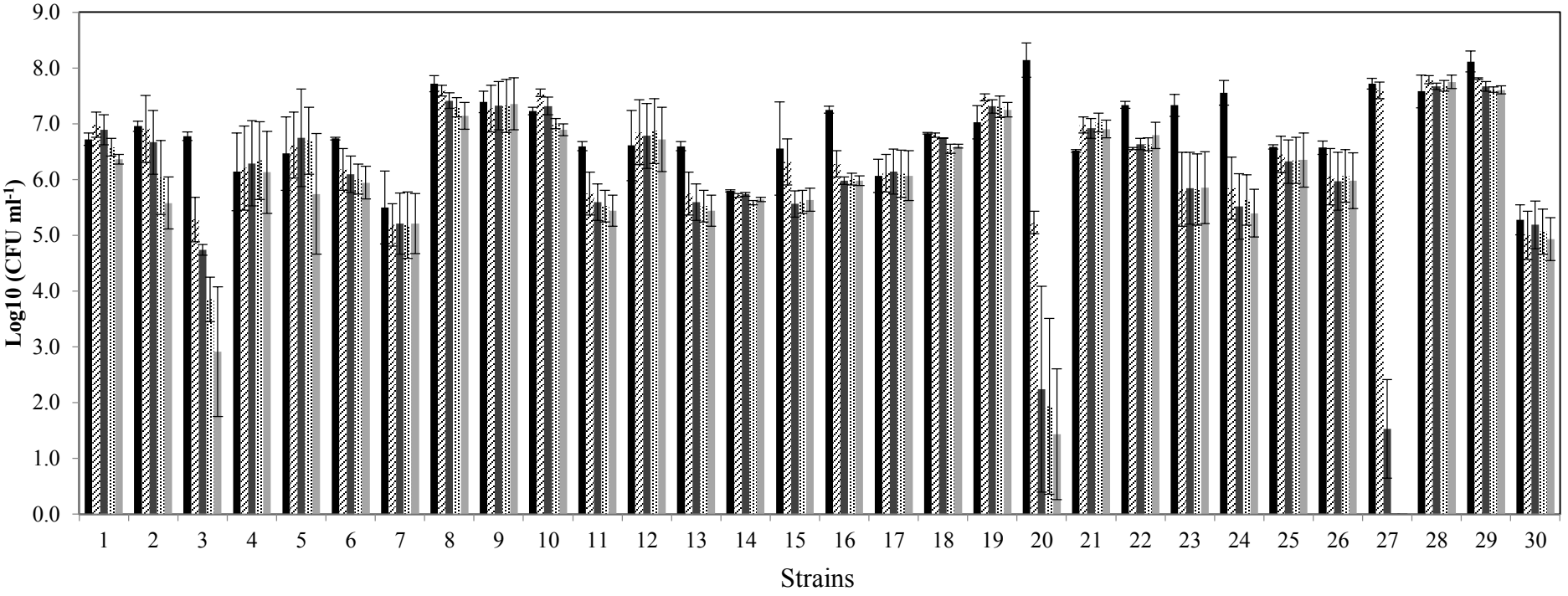

C

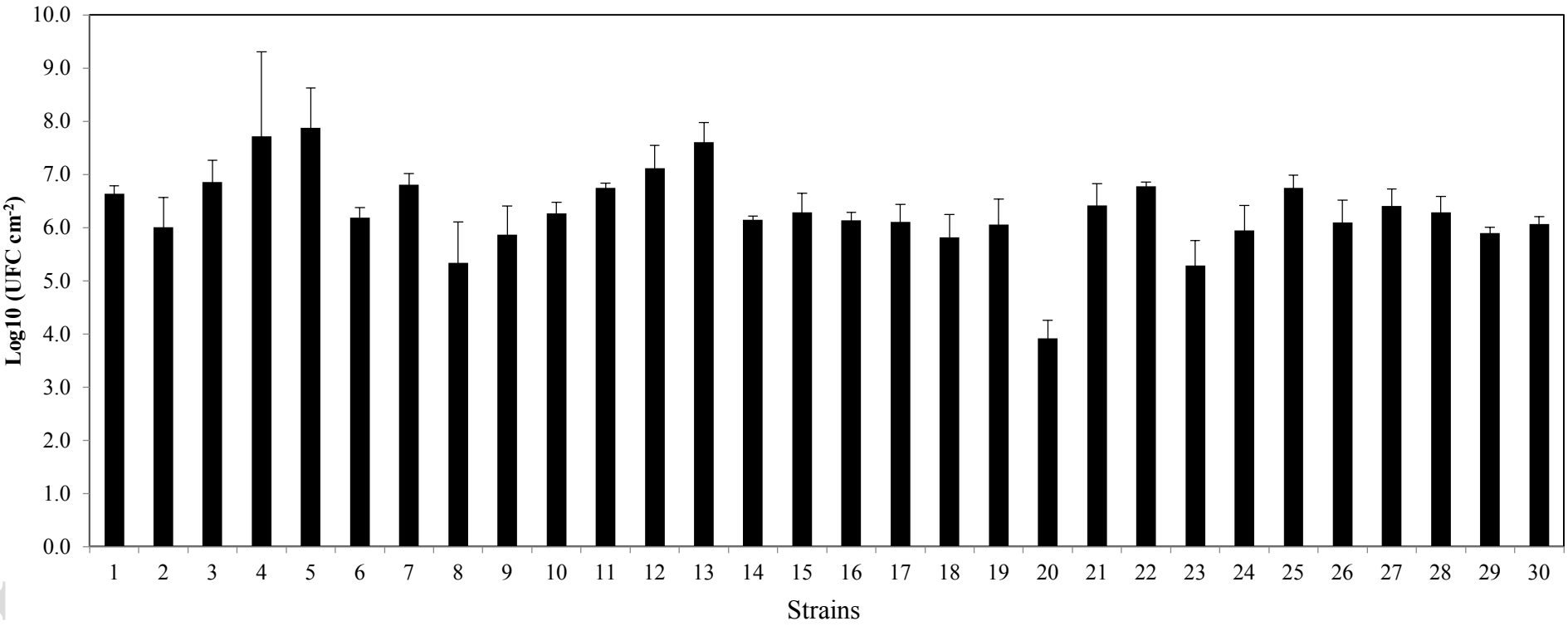

Fig.3